IN THE UNITED STATES PATENT AND TRADE MARK OFFICE

DECLARATION UNDER 35 U.S.C §1.132

Sir:

I, Gerald Wayne Both am a co-inventor of United States of America Patent Application No 09/464767, entitled "DNA Encoding Ovine Adenovirus (OAV287) And Its Use As A Viral Vector" (the Application). My curriculum vitae has been previously filed on 16 December 1999 with respect to United States of America Patent Application No 09/464767.

I have supervised and carried out further experiments relating to the ovine adenovirus of the invention claimed in US 09/464767. Theses further experiments are described below.

Additional plasmids containing the infectious OAV genome were constructed using standard molecular biology techniques (9) and new viruses have been rescued. These further demonstrate the utility of OAV as a gene delivery vector. Figure 7 of the Application shows plasmid pOAV600 in which unique ApaI/NotI cloning sites were inserted into a non-essential region of the genome. This plasmid was further modified. Nucleotides between the NotI and SalI sites (Figure 7) were deleted by digestion with these restriction enzymes and replaced by cloning in the ~82bp sequence located between the NotI and SalI sites of the plasmid Bluescript KS$^+$ (Stratagene, La Jolla, CA). This deleted 1938 nucleotides from the OAV genome and created plasmid pOAV603 (Figure 13). Plasmid pOAV209 (Figure 13) was constructed by subcloning the XbaI-cut pACYC184 (4,244bp) into the XbaI site of Bluescript KS$^+$. The fragment was then excised using flanking ApaI and NotI sites and subcloned into ApaI/NotI-cut pOAV200 (Figure 7). Plasmids pOAV209 (Amp^R/Cm^R) and pOAV603 were characterised by digestion with restriction enzymes. pOAV287Cm (Figure 6) and pOAV209 contain the same ~4.2kb "stuffer" DNA fragment from pACYC184. For the former the fragment was cloned into the unique SalI site of the OAV genome. Plasmid pOAV623 contains a gene cassette comprising an enhancer element derived from the prostate specific membrane antigen gene linked to the rat probasin promoter and the purine nucleoside phosphorylase gene derived from *E. coli*. This cassette of total size ~2.4kb was cloned into the ApaI/NotI sites of pOAV600 in the left to right orientation. Based on

the transcription map of OAV that we have determined (Khatri and Both, 1998) the cassette in pOAV623 is located between the transcription units for the left end and proposed E4 regions of the OAV genome. In addition, a cassette comprising the Rous Sarcoma virus LTR linked to the human alpa-1-antitrypsin cDNA and bovine growth hormone polyadenylation signal was cloned into the ApaI/NotI sites of pOAV200 in the right to left orientation (pOAVhAAT, Figure 13), which is the opposite orientation compared with all other cassettes. These plasmids were digested with KpnI or its isoschizomer, Asp718 to release the linear genome. The DNAs were individually transfected into CSL503 cells using methods described at page 13, line 5-23 of the Application. The viruses corresponding to the plasmids described in Figure 13 were rescued, characterised by restriction enzyme digestion and shown to have the correct structure.

The rescue of these viruses identifies additional useful properties of the OAV vector. Two new non-essential sites for the insertion of foreign DNA have been identified. The ApaI/NotI cloning sites in pOAV600 (Figure 7) have been shown to be suitable for the construction of a recombinant virus by the rescue of OAV623 (Figure 13). The SalI site near the right end was identified in pOAV600S (Figure 7) as non-essential (page 19, line 34-page 20, line 6 of the Application). It was then realised that the foreign DNA DNA (from pACYC184) carried by pOAV287Cm (Figure7) was inserted in a non-essential region. This was confirmed by the fact that the corresponding virus, OAV287Cm, was rescued (Figure 13). A similar virus (OAV209, Figure 13) in which the same pACYC184 DNA was inserted between the pVII and fiber genes was also rescued. Thus, these data show that the OAV capsid can package at least 4.23kb of foreign DNA i.e. 114% of the wild type genome, in either of two sites without a compensation deletion. This capacity is significantly greater than that of human adenovirus type 5 (Bett *et al.*, 1993) and is large enough to accommodate many individual genes of interest to researchers.

In addition, ~2kb of non-essential sequences have now been identified in the OAV genome. Surprisingly, four of six potential open reading frames in the transcription unit designated as RE in Figure 13 are closely related to each other and may have redundant functions (Xu *et al.*, 1997). In confirmation of this, deletion of all but one of these reading frames in pOAV603 still allowed the rescue of a viable virus. Thus, deletion of these sequences coupled with the ability to insert at least 4.23kb of DNA means that OAV has capacity to carry at least 6.3kb of foreign DNA.

Vaccination *in vivo*

The construction of recombinant virus OAV205 (Figure 10) is broadly described on page 21 lines 19-33 of the Application. More specifically, pOAV205 was constructed by subcloning a gene

fragment encoding the 45W antigen from the sheep parasite *Taenia ovis*. The gene fragment, excised as an EcoRI/BamHI fragment, was linked to the OAV MLP/TLS sequences (page 21, lines 13-21 of the Application) in plasmid pMT (Figure 9) cut with HindIII/BglII, via an HindIII/EcoRI adapter. The adapter also provided an in-frame ATG codon. The MLP/TLS/45W cassette was excised as an ApaI/NotI fragment and subcloned into pOAV200 (Figure 10). The virus expresses the antigen known as 45WB/X in CSL503 cells infected in vitro (Figure 11A). This virus was used to vaccinate sheep, alone, or in combination with a purified plasmid, pcDNA3-45W (Rothel *et al.*, 1997) that expressed the 45W antigen, or with purified 45W-GST fusion protein that had been expressed in *E. coli* (Lightowlers *et al.*, 1996). Dorset X Merino wethers and ewes that were 10-12 months of age at the time of first vaccination were inoculated intramuscularly with plasmid (200ug/dose in a total volume of 1ml of phosphate-buffered saline), 45W-GST (50ug/dose formulated with Quil A adjuvant (Superfos, Denmark; 1mg/dose). For both primary and secondary inoculations wild-type OAV or recombinant OAV205 was given intra-muscularly (~$2x10^6$pfu), intra-nasally (~$3.4x10^7$pfu) and intra-conjunctivally (~$1.7x10^7$pfu). There was a 4-week gap between primary and secondary inoculations. Antibody formation (classes IgG_1 and IgG_2) against 45W protein was detected by ELISA (Rothel *et al.*, 1996).

In a pilot experiment, two of three sheep inoculated with OAV205 developed low levels of 45W-specific serum antibody titres (<200), whereas specific antibody could not be detected in sera from animals inoculated with wild type OAV. In a larger experiment animals were vaccinated as shown in Table 1. Sheep vaccinated with two doses of either the plasmid DNA or OAV205 vaccines elicited low levels of 45W-specific antibody with the adenovirus vector proving more effective (Figure 14). Animals vaccinated with OAV205 mounted predominantly an IgG_1 response that was maximal two weeks after the primary vaccination. This was not further boosted by re-vaccination at 4 weeks. For all sheep that received the OAV205, low levels of OAV-specific antibody were detected in sera collected two weeks after the second vaccination. The mean OAV-specific IgG titre for these animals was 548 (+/-486) with a range of 120-2000.

Although animals vaccinated with OAV205 elicited low levels of 45W-specific antibody they were primed to generate an enhanced immune response after subsequent vaccination with 45W/Quil A (Figure 15, compare B and D with A). The IgG_1 titres for D are significantly higher than for A (Mann-Whitney U=17, P<0.05). Similarly, vaccination with OAV205 can boost an immune response primed with protein/Quil A (Figure 15, compare A with C).

In addition, an immune response primed by vaccination with plasmid DNA is boosted by OAV205. Animals from group 9 (Table 1) generated high levels of 45W-specific antibody that was predominantly of the IgG_1 isotype. All three sheep had low but detectable titres after the second DNA vaccination (titres of 110, 140 and 150). One week after vaccination with OAV205 the mean IgG_1 titre was 9367, a level >65 fold higher than that in sheep receiving two inoculations of either DNA or OAV205 alone and comparable to the levels achieved with two inoculations of protein/Quil A (mean titre 11833).

A challenge experiment was carried out to ascertain whether the antibody levels generated by the various vaccination procedures were protective against *Taenia ovis*, the parasite from which the 45W antigen was derived. Eight weeks after their final vaccination animals from groups 1, 2, 3, 9 and 10 (Table 1) were challenged by intra-ruminal injection of *T. ovis* 5000 eggs. A further four weeks later these sheep were killed and their heart, diaphragm, masseters and the muscle tissue of their right rear legs (vaccination site) was examined for the presence of cysticerci by slicing the tissues at ~3mm intervals.

Sheep sequentially immunised with plasmid DNA and boosted with OAV205 were significantly protected from *T. ovis* challenge (Table 1) (Mann-Whitney U=19, P=0.02). Similarly, sheep initially vaccinated with protein/Quil A (group 2) were protected (U=17.5, P, 0.05). As a group the animals that received two inoculations with protein/Quil A (group 1) were not significantly protected (P=0.078). This was probably a reflection of the 45W-specific antibody levels in two of these sheep that dropped to non-protective levels by the time of challenge. In addition, the two surviving sheep in group 3 (Table 1) had only low 45W-specific antibody levels at the time of challenge and were not protected. Overall there was a significant inverse correlation between 45W-specific IgG_1 titres and cyst numbers (Pearson correlation P<0.05). However, there was no correlation between cyst numbers and IgG_2 levels (P=0.152).

In summary, these data show that the ovine adenovirus vector OAV205 is capable of delivering a gene for a parasite antigen to sheep such that a measurable immune (antibody) response is induced. Further, the recombinant vector primes an immune response that can be boosted by vaccination with the recombinant protein. The vector can also be used to boost an immune response primed by 45W plasmid DNA or protein/Quil A vaccine. Combination vaccination strategies are able to induce a protective immune response against a sheep parasite.

I further declar that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements are made with the knowledge that wilful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of the Title 18 of the United States Code, and that such wilful false statements may jeopardise the validity of this application or any patent issuing thereon.

Dated: 18/03/03

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1. Additional references

Bett, A. J., Prevec, L., and Graham, F. L. (1993). Packaging capacity and stability of human adenovirus type- 5 vectors. *J Virol* 67(10), 5911-5921.

Khatri, A., and Both, G. W. (1998). Identification of transcripts and promoter regions of ovine adenovirus OAV287. *Virology* 245(1), 128-141.

Lightowlers, M. W., Waterkeyn, J. G., Rothel, J. S., Gauci, C. G., and Harrison, G. B. (1996). Host-protective fragments and antibody binding epitopes of the Taenia ovis 45W recombinant antigen. *Parasite Immunology* 18(10), 507-13.

Rothel, J. S., Lightowlers, M. W., Seow, H. F., Wood, P. R., Rothel, L. J., Heath, D. D., and Harrison, G. B. (1996). Immune responses associated with protection in sheep vaccinated with a recombinant antigen from Taenia ovis. *Parasite Immunology* 18(4), 201-8.

Rothel, J. S., Waterkeyn, J. G., Strugnell, R. A., Wood, P. R., Seow, H. F., Vadolas, J., and Lightowlers, M. W. (1997). Nucleic acid vaccination of sheep: Use in combination with a conventional adjuvanted vaccine against Taenia ovis. *Immunol Cell Biol* 75, 41-6.

Xu, Z. Z., Hyatt, A., Boyle, D. B., and Both, G. W. (1997). Construction Of Ovine Adenovirus Recombinants By Gene Insertion And Deletion Of Related Terminal Sequences. *Virology* 230, 62-71.

Figure legends.

Figure 13. Structure of OAV genomes in several new recombinant viruses. The viruses were rescued from the corresponding plasmids in which sequences from a modified Bluescribe plasmid linked the ends of the viral genome as shown in Figure 7 and described on p12, lines 23-36.

Figure 14. Mean 45W-specific IgG_1 (circles) and IgG_2 (squares) ELISA titres of sheep vaccinated with either pcDNA-45W (diamonds) or recombinant OAV205 (squares). The three sheep per group were vaccinated at week 0 and revaccinated at week 4.

Figure 15. Mean 45W-specific IgG1 (solid bars0 and IgG2 (hatched bars) ELISA titres of sheep vaccinated with various combinations of vaccine delivery systems. Values are the mean titres (with standard errors) for each vaccine group measured in sera collected two weeks after the last vaccination. Animals were vaccinated as follows; A, protein/Quil A; B, protein Quil A twice, four weeks apart; C, protein Quil A, followed by OAV205; D, OAV205 followed by protein/Quil A.